

Advantages of Using Aquatic Animals for Biomedical Research on Reproductive Toxicology

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Major advantages of the use of aquatic animals, such as trout, English sole, or sea urchins, for studying the mechanisms of reproductive toxicology are discussed. The remarkable synchrony of differentiation of gametes in large quantities for detailed morphologic and biochemical measurements enables research not readily done on mammalian nonseasonal breeders. Structural differences such as the absence of a fibrous sheath in the more simple structure of fish and sea urchin sperm flagella facilitates comparative study of the mechanism of action of microtubules in flagella movement and the coupling of mitochondrial energy production to microtubules movement.

Introduction

Solutions for two opposite but persistent human reproductive problems—undesired pregnancy and infertility—hinge on our knowledge of biologic mechanisms controlling gametogenesis. From the beginning of microscopical science (1) Leeuwenhoek recognized organelles in spermatozoa. Continuing to the present, scientists have appreciated the central role of gametogenesis as the final common pathway in reproductive physiology. The early biologists made use of many species of animals for the study of reproduction, including frogs, chicks, fish, and mammals, whichever seemed to them the most propitious. The use of humans and laboratory rodents as experimental animals has far overshadowed the use of all other species for reproduction research in recent decades (2,3).

Our objective is to re-emphasize some of the advantages of the use of aquatic species for current research problems on the mechanisms of injury to gametogenesis by toxic chemicals. The comments will be limited to spermatogenesis. Future studies must be directed toward gaining a better understanding of the complex processes controlling proliferation and maturation of sperm and the defects responsible for specific structural or functional failure of this process. Several aquatic species offer unique opportunities for this research. We

shall use spermatogenesis in English sole (*Parophrys vetulus*), rainbow trout (*Salmo gairdneri*), and sea urchins (*Strongylocentrotus purpuratus*) for comparison with that of higher mammals. Of course, some aquatic species are not seasonal breeders and are not appropriate for this discussion.

There are several practical advantages for using these aquatic animals for the study of reproductive biology problems: (1) they are inexpensive; (2) they produce massive numbers of sperm (and ova) which can be readily obtained for analysis; (3) the remarkable synchrony of differentiation increases "signal over noise" in morphologic, functional, and biochemical studies; (4) morphologic similarities and differences with mammals can be exploited; (5) by selection of species, short or long generational cycles can be used; (6) being oviparous, fertilization and the early stages of development can be studied in a natural ecosystem without artificial manipulation in the laboratory. For example, superovulation of a mammal in the laboratory may produce a dozen or so ova for fertilization, early embryogenesis, or toxicology studies, whereas a fish or sea urchin would provide thousands of zygotes.

There are also some disadvantages to selecting an aquatic species for reproduction research: (1) preliminary background research data are usually very sparse, and thus often one must begin by doing numerous baseline studies on dose-response, controlled experimental conditions, etc., before beginning experiments on the major hypothesis; (2) facilities for the culture of large fish and personnel for their care are not as widely avail-

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able as are those for mammals (4). However, sea urchins and smaller fish such as the medaka can be easily reared in the laboratory at minimum expense.

Spermatogenesis; Comparative Histology

Spermatogenesis is an elaborate cytological process by which stem cells (spermatogonia) are transformed into mature spermatozoa. The process of spermatogonial proliferation and differentiation into spermatozoa lasts for about 5 to 8 weeks in various mammalian species, occurring in the seminiferous tubules of the testes and accessory organs such as the epididymis and seminal vesicles. In general, less research is being done on the formation of the spermatozoan and the effects of toxic chemicals on the process than on the biochemical composition of semen associated with the modification of the fully formed spermatozoa as it travels through the male and female reproductive tracts.

Spermatogenesis can be divided into three phases, each having well-characterized morphologic features. The first phase is the proliferation of spermatogonia, some of the daughter cells of which give rise to spermatocytes whereas the remainder maintain the number of spermatogonia by continued proliferation. The second phase involves the differentiation of primary and secondary spermatocytes that go through a process of reduction division (meiosis) converting the diploid number of chromosomes to the haploid number in the spermatids. The third phase involves the conversion of the spermatids by means of subtle and complex biochemical processes leading to the production of the activated sperm cell. The maintenance of spermatogonial stem cells is directly related to the maintenance of male fertility because they provide renewal that is essential for the continued production of spermatozoa. A decrease in the population of these stem cells may result in sterility due to insufficiency of sperm. Thus, chemical and physical agents that are deleterious to cell division (mitosis) can be expected to have an adverse effect on sperm count.

Spermiogenesis is a term applied to the transformation of newly formed spermatids through a largely unidentified complex series of biochemical changes leading to the production of highly differentiated motile spermatozoa.

In the aquatic species we describe here, the major cellular morphologic and biochemical features of the three phases of differentiation are remarkably similar to that in humans, but there is a recognizable species specificity to the structure of sperm. Primitive metazoa up the phylogenetic scale through teleosts have sperm of simple structure (5). These species usually discharge semen for external fertilization. Animal species with internal fertilization have spermatozoa with a more complex morphology. Several reports have established that the major morphologic features of rainbow trout (*Salmo gairdneri*) spermatogenesis are similar to those in mam-

mals (6-9). However, an important difference is that rainbow trout and English sole are seasonal breeders, and this provides research advantages over the use of nonseasonal breeder such as mammals, including humans and primates. For example, in English sole caught in June in Puget Sound the seminiferous tubules are quiescent with little or no proliferation of spermatogonia (Figs. 1 and 2). In September and November, prior to spawning in February, the spermatogonia rapidly proliferate and differentiate synchronously in all tubules (Fig. 3) and at the time of spawning in February the cells are almost exclusively spermatozoa (Fig. 4). By contrast, in the nonseasonal breeders the several processes progress asynchronously at different locations and in different seminiferous tubules (Figs. 5 and 6), with some of the cells concomitantly undergoing degeneration and necrosis (10,11). Thus moderate effects of a toxic substance may be masked. With synchronous spermatogenesis, however, there is little necrosis; thus fewer samples and less extensive morphometric quantitation are needed to detect effects. Prior to natural spawning, trout testes contain less than 2% spermatids and spermatozoa; during spawning the numbers of these differentiated forms rapidly increase to above 95% of the cells of the testes (9). After releasing the sperm the

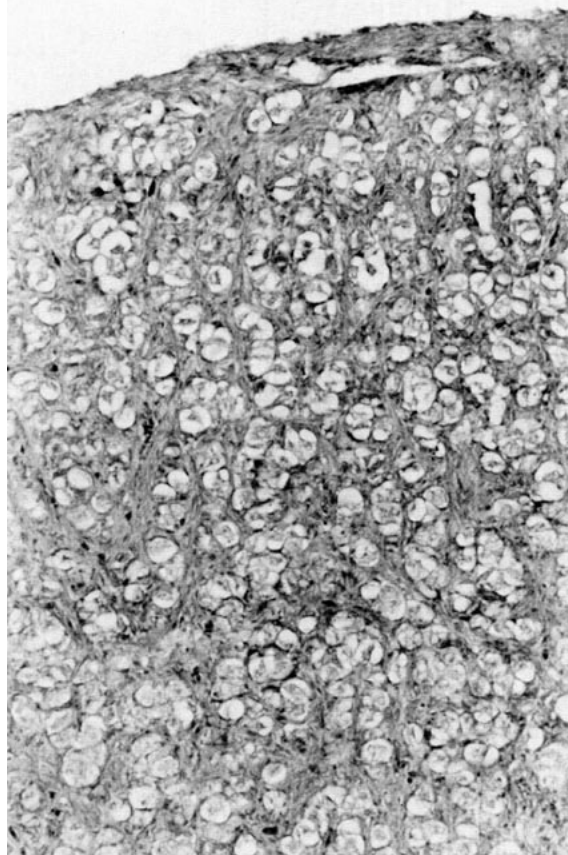


FIGURE 1. Photomicrograph of testis. Quiescent testis with spermatogonia filling tubules between fibromuscular stroma. English sole M81, 302 g, June. $\times 160$.



FIGURE 2. Photomicrograph of testis. Proliferated spermatogonia are the dominant cell type. Morphologic uniformity throughout. English sole E7, 292 g, September. $\times 50$.

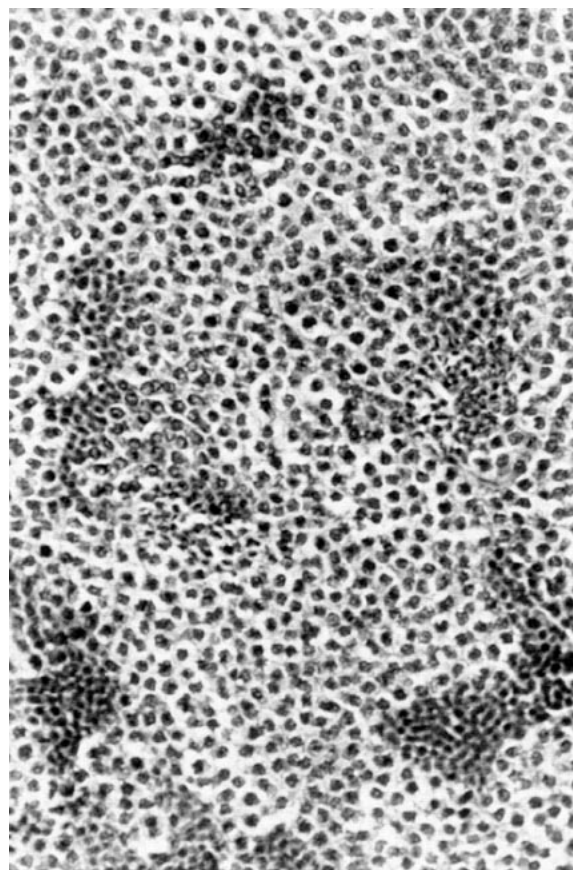


FIGURE 3. Photomicrograph of testis. Largely synchronous field of spermatocytes with a few foci of early spermatid differentiation. English sole T26, 289 g, November. $\times 160$.

spermatogonia and Sertoli cells again represent more than 98% of the cells of the testes (12).

Spermatogenesis can be induced in the rainbow trout testes with gonadotropic hormone (GH) injections as described by Drance et al. (6). The rate of acceleration of differentiation depends on hormone dose and experimental conditions. Drance and co-workers described an orderly progression and found that large numbers of cells at the same stage of development were seen when sampled each week. They further stated that there was no significant difference in morphology in sperm cells in the accelerated system in comparison with normal. Similar results have been obtained by using other species of Salmonids (13–15). Obviously the maturity of the fish, dose of the hormone, photoperiod, temperature, pH, and experimental conditions can alter the rate of maturation (14,16–21) and can provide samples for study in the laboratory virtually year round (6,13,19).

The synchrony of differentiation in the testis facilitates morphologic procedures such as flow cytometry. This procedure requires that the cells are synchronous in order to measure ploidy or to investigate the cell cycle (22–25). Serum deprivation or contact inhibition are frequently used to make cultured cells synchronous. The naturally synchronous differentiation of fish male ga-

metes simplifies the measurement of ploidy in these cells. Another advantage of the seasonal breeder is that there is a virtual absence of degenerating spermatogenic cells at the time of stimulation. This is in contrast to mammalian species, where up to 25% of the cells may die during spermatogenesis (10,11,26–28). The presence of such high levels of “physiologically” necrotic cells increases the “noise” level and complicates identification of the effects of toxic chemicals except in extreme cases.

Sperm Flagellar Substructure and Function

Major gaps in our knowledge of the fundamental mechanism of sperm motility include: the manner by which the dynein ATPase transforms the chemical energy released by hydrolases of ATP into mechanical energy of sliding tubules (2,12); the coordination mechanisms responsible for regulating the sliding between tubules to produce a propagated bending wave; the nature of major protein components of mammalian sperm flagella; and the function of the supplementary extra axonemal structures in sperm flagella. Comparative studies between the lower forms that have simpler flagella and the complex ones that have extra axonemal

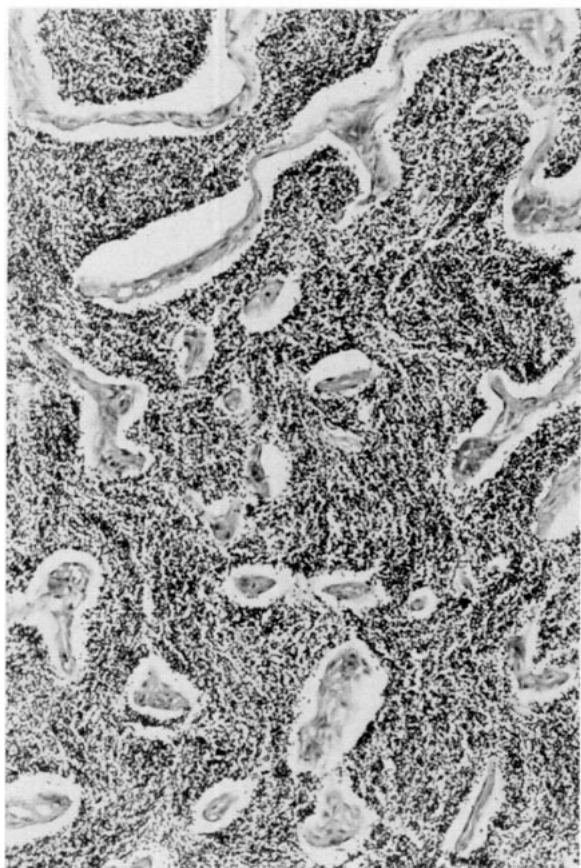


FIGURE 4. Photomicrograph of testis. Uniform population of spermatozoa fills interstices between stroma. R7, 300 g, February (prior to spawning). $\times 50$.

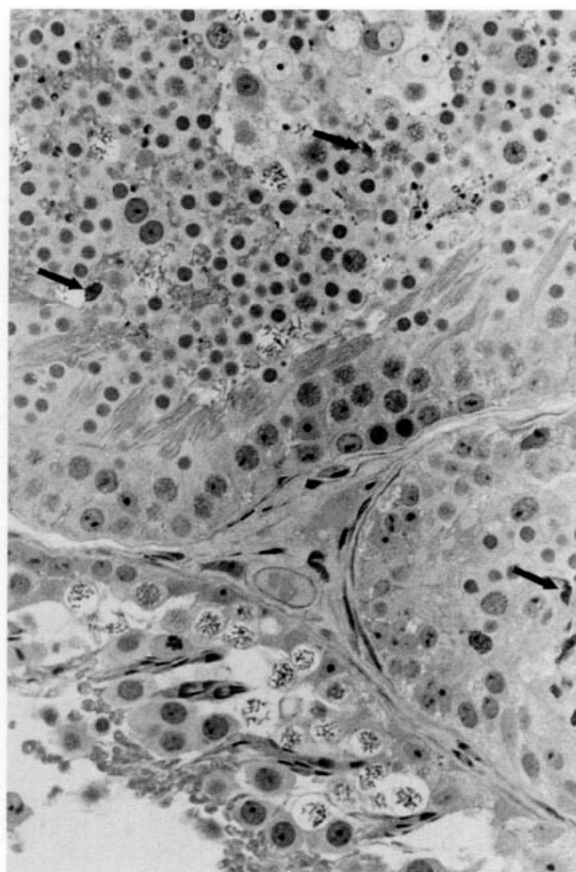


FIGURE 6. Adult *M. mulatta* testis. All stages from spermatogonia to spermatids seen in one microscopic field. Arrows denote necrotic cells. $\times 160$.

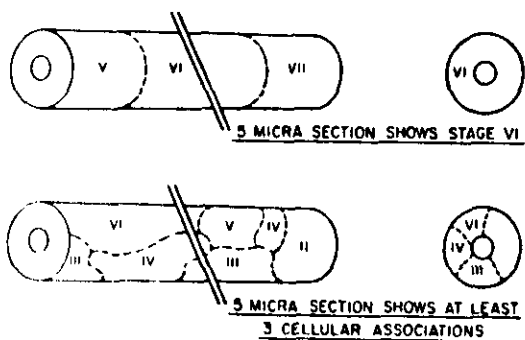


FIGURE 5. Schematic drawing of seminiferous tubules, of the cell associations in most subhuman species (top) showing a major difference in the organization and man (bottom). In subhuman species each cell association occupies a more extensive area along the length of the tubule (43).

structures could provide an excellent tool for characterizing these functions.

We shall illustrate these advantages by discussing sperm motility research in greater detail. Sperm structure separates functions very precisely: the sperm head packages the male contribution to the genome, and the

flagellum is the propulsive structure. The internal structure of the flagellum contains an axoneme made up of microtubules with a $9+2$ pattern of filaments running throughout its length. In fish and sea urchin spermatozoa the simple flagellum consists of the axoneme surrounded by a cell membrane (29). Spermatozoa characteristic of animals with internal fertilization have flagellas of a more complex structure in which the $9+2$ axoneme of the simple flagella is supplemented by an additional fibrous sheath (29). By selection of species one can "dissect" the effects of toxic agents on these components of the flagellum. The microtubules of the axoneme have dynein arms arranged alongside each microtubule and radial spokes connecting adjacent tubules (12,30,31). One of the important remaining problems concerning sperm motility is how these arms generate sliding movements to produce the bending of the flagellum. Perhaps this type of investigation could best be done on flagellas lacking the fibrous sheath.

An example of research on sperm function is currently under way in one of our laboratories (NKM) involving the toxicity of methylmercury on sperm. The research began with the use of primates and has led us to consider the use of aquatic animals. Our first step in examining the toxicity of methylmercury toward male reproduc-

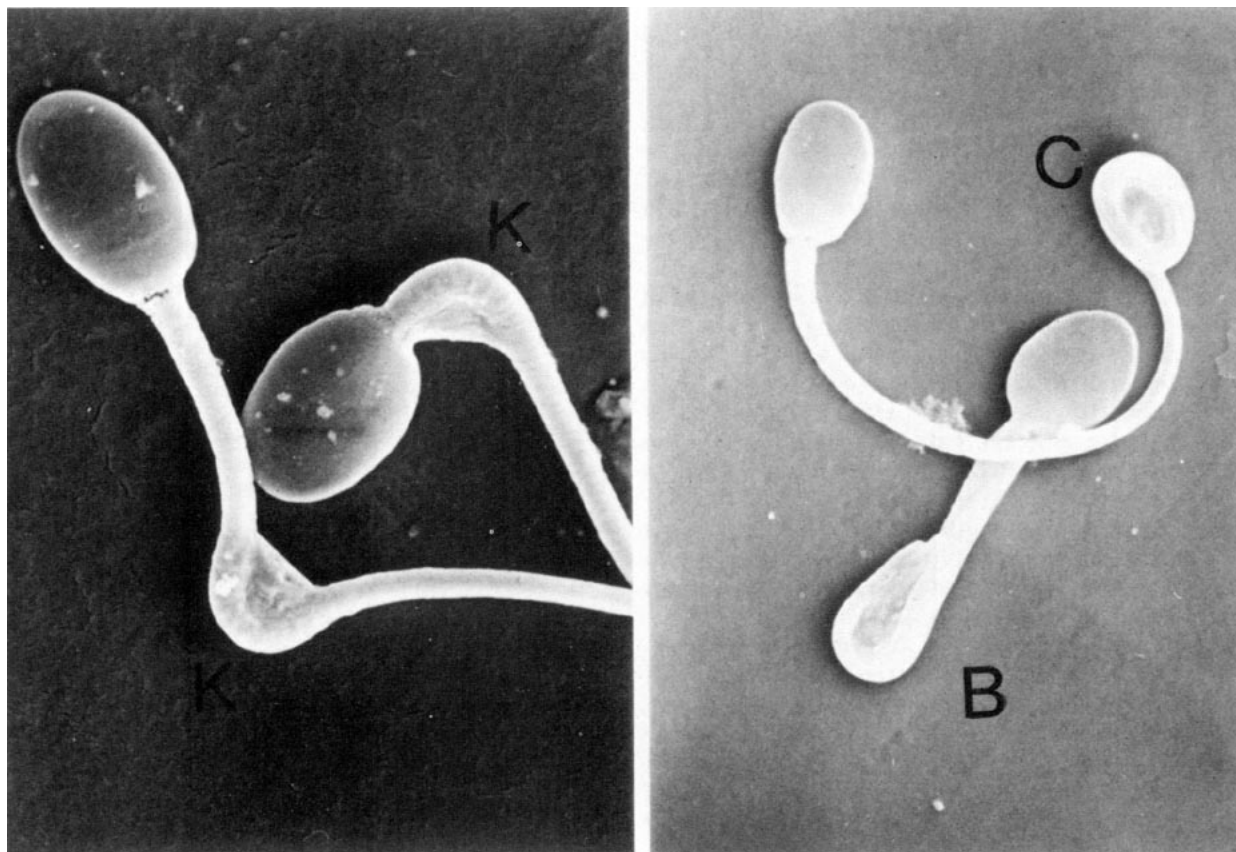


FIGURE 7. Scanning electron micrograph of *M. mulatta* sperm following methylmercury exposure: K = kinks; C = coils; B = bends of tails.

tive function was to establish that there was indeed an effect in primates (32,33). The three major epidemics of mercury poisoning in the world plus numerous individual case reports were associated with over 8500 people being hospitalized and with more than 580 deaths. However, no evidence of male or female reproductive impairment due to exposure to methylmercury was reported. This is perhaps understandable due to the nature and duration of the exposures in the human populations and to the "field" nature of the studies. There was, however, one letter to the editor pertaining to an occupational exposure to mercury in which decrease in spermatogenesis and fertility were noted. Several rodent experiments indicate that methylmercury does indeed decrease fertility in the male (34).

To determine whether methylmercury had an effect on sperm we first exposed primate semen to 1, 2, 3, etc., ppm of methylmercury *in vitro* and observed a remarkable decrease in swim speed as revealed by laser methods (35). The sperm swim speed was reduced to 69% of control at 2 ppm methylmercury. An *in vitro* assay of oxygen uptake by sperm exposed to methylmercury was compared with the controls. Oxygen uptake was altered at 9 ppm and above, but no effect was seen at lower doses, even though the sperm speed was decreased (36). Therefore changes in energy production were not involved in the decreased sperm swim speed.

Something inherent in the structure of the flagellum must have been changed.

Light microscopy revealed kinking and coiling of sperm (Fig. 7) that were exposed either *in vitro* or *in vivo* to methylmercury. Approximately 40% of the sperm were so altered with a blood level of 2 ppm methylmercury. Flagellar deformities in these sperm led us to consider the microtubules as the site of injury by methylmercury. As noted above, the axoneme of the flagellum is a microtubular structure. Also, assembly of microtubules is very important in the manchette of the Sertoli cells and in the mitotic spindles of the spermatogonia.

Microtubules alluded to above are long, relatively rigid, unbranched polymers of the subunit proteins, alpha and beta tubulin, and are ubiquitous components of eukaryotic cells intimately associated with many important cell motility processes. Much of our knowledge of the assembly of the axoneme of cilia and flagella has been derived from studies on organisms from protozoa to vertebrates (31,33,37,38). Morisawa and Mohri (39) studied the formation of axoneme in sea urchins. Also in our laboratory (40) we have studied the effects of methylmercury binding to microtubules and have shown that *in vitro* polymerization of microtubules was totally inhibited at 3.0×10^{-5} M MeHg, and depolymerization occurred at concentrations above 1.0×10^{-5} M MeHg.

We demonstrated that there are 15 free sulfhydryl groups per tubulin dimer and that MeHg can bind to all 15. However, when MeHg binds only two free sulfhydryl groups per dimer, it inhibits polymerization. MeHg bound the free sulfhydryl groups at the ends of the microtubule as well as those on the surface. Thus, our current hypothesis is that the deformity of the sperm flagellum is due to a defective positioning of the microtubules in portions of the axoneme.

The cost and time needed to test this hypothesis in mammals would be excessive, so the logical next step is to use an aquatic species. This will provide us with synchronized spermatogenic cells and provide us with numerous opportunities to both ultrastructurally and functionally determine the nature of the injury to the sperm. As the axoneme lacks the outer dense fiber coat seen in mammals, the effects of a toxic agent directly on the axoneme can be contrasted with the functional activity of the dense fiber layer. The changes shown above occurred mainly in areas where the axoneme was not covered by the outer dense layer.

The morphologic advantages of having massive numbers of synchronous cells also pertain to biochemical studies of sperm flagellar function. A brief review such as this cannot do justice to the elegant studies at our university by Shapiro and co-workers (41,42) using sea urchin sperm. Capitalizing on the massive numbers of synchronous cell populations, they were able to readily manipulate them *in vitro*. Sea urchin sperm provide an excellent system in which to examine cellular regulation. For example, the utilization of energy by sperm is strictly coupled to its rate of production. This tight coupling depends on transport of high energy phosphate from mitochondrion to axoneme which appears to be mediated by a phosphorylcreatine shuttle. Thus, inert quiescent sea urchin sperm can be induced to maximum swimming activity and reversed in 10^{10} sperm at will. This is a highly programmed response with no concomitant DNA synthesis or other cellular activities. All the cells are doing only one thing. Therefore, the "signal to noise" ratio is extremely high. By increasing the acidity of the medium 0.5 pH the sperm activity response increases 50-fold.

These are a few of many examples of how the study of reproductive toxicology can be facilitated by the use of aquatic animals. It appears to us that the relationship of axoneme structure in the flagellum, the dynein sidearms and energy utilization in the flagellum, and the site of toxic injury to sperm motility can best be studied in aquatic species.

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